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Characterization and Distribution of *Pasteurella* Species Recovered from Infected Humans

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During a 3-year period, all *Pasteurella* strains recovered at the Clinical Microbiological Laboratory, Lund, Sweden, were studied biochemically with respect to their relationship to the recently described taxa of this genus. Of 159 strains recovered from 146 infected humans, 95 were identified as *Pasteurella multocida* subsp. *multocida*, 21 as *Pasteurella multocida* subsp. *septica*, 28 as *Pasteurella canis*, 10 as *Pasteurella stomatis*, and 5 as *Pasteurella dagmatis*. The homology within and between the *Pasteurella* species regarding cellular fatty acids and enzymatic activities was also studied. Strains of the different *Pasteurella* species were indistinguishable from each other regarding fatty acid composition; all strains contained major amounts of $C_{14:0}$, $C_{16:1}$, $C_{16:0}$, and 3-OH-$C_{14:0}$ acids and minor amounts of $C_{18:2}$, $C_{18:1}$, and $C_{18:0}$ acids. Neither did the enzymatic activities distinguish between strains belonging to different species. In addition, of 56 strains examined, toxin production was demonstrated only in 1 strain each of *P. multocida* subsp. *multocida* and *P. canis*. Except for one severe case of necrotizing cellulitis involving *P. dagmatis*, *P. multocida* subsp. *multocida* or *P. multocida* subsp. *septica* was recovered in the more serious cases of infection. Except for *P. canis*, which in all cases was associated with dog bites, most *Pasteurella* strains were recovered in cases of infection associated with cat bites or scratches. *Pasteurella* strains occurred in four infected patients without evident connections with animals.

Pasteurella species, which occur as indigenous organisms in the oral and gastrointestinal floras of many wild and domestic animals, are important pathogens in both animals and humans. Human infections caused by *Pasteurella* species are in most cases associated with infected injuries following animal bites (8, 19). However, severe *Pasteurella multocida* infections occur also in the absence of animal bites or scratches. In most of those cases, licking of intact or injured skin by pet animals was the likely source of entry for the organism (9, 16, 17). Infections associated with inhaled microorganisms, or with no known source of acquisition, have also been reported (11, 12, 19).

Recently, a reclassification of the genus *Pasteurella* was presented (13). On the basis of DNA hybridization studies, 13 taxa, including the new species *P. canis*, *P. stomatis*, *P. dagmatis*, *P. anatis*, and *P. langaa*, were detected (13). *P. canis* comprises strains previously classified as *P. multocida* biotype 6, or "dog type" (4), and *P. dagmatis* contains organisms previously designated *Pasteurella* "gas," *Pasteurella* new species 1 (5), and *P. pneumotropica* type Henriksen (6). *P. multocida* now includes three subspecies, *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, and *P. multocida* subsp. *gallicida*.

In order to investigate the distribution of *Pasteurella* species in human infections, we characterized all *Pasteurella* strains recovered in our laboratory during a 3-year period with respect to their relationship to the recently described taxa. The homology within and between the different species regarding enzymatic activities, toxin production, and cellular fatty acid composition was also studied.

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MATERIALS AND METHODS

Bacteria. During a 3-year period (between February 1989 and March 1992), all *Pasteurella* strains recovered from clinical specimens submitted for microbiological diagnosis to the Clinical Microbiology Laboratory, University Hospital, Lund, Sweden, were studied. Altogether, 159 strains were isolated from 146 specimens in 146 cases of infection. Four strains were isolated in cases of wound infection and a case of an abscess in patients without evident connections with animals, while 155 strains were recovered in cases of infection associated with cat bites, scratches, or licking (87 infections) or with dog bites or licking (55 infections), comprising 147 strains isolated from wound infections or abscesses, 5 strains from blood specimens from patients with septicemia, 2 strains from cerebrospinal fluid of patients with meningitis, and 1 strain from peritoneal fluid from a patient with peritonitis.

The strains had been identified as *Pasteurella* strains when shown to be nonmotile, gram-negative coccobacilli which were nonhemolytic when incubated on sheep blood agar and positive for indole (with a few exceptions), catalase, and oxidase (Kovács reagent spot test). All strains produced acid from glucose and sucrose and were lysine negative.

Biochemical tests for differentiation of *Pasteurella* species and subspecies. Acidification of carbohydrates was tested in phenol red carbohydrate media (Difco, Detroit, Mich.) to which one of the following carbohydrates had been added according to the manufacturer's instructions: arabinose, dulcitol, glucose (to test for gas production), maltose, mannitol, sorbitol, trehalose, and xylose. The carbohydrate media were incubated for up to 4 days at 37°C. Other tests included decarboxylation of ornithine, indole production (spot test), nitrate reduction, hydrolysis of urea, liquefaction of gelatin, and tellurite tolerance.

Enzymatic activities. Enzymatic activities were determined with API-Zym (API-System, La Balme les Grottes,

TABLE 1. Biochemical differentiation and distribution of 159 strains of *Pasteurella* isolated from infected humans

Test	No. (%) of positive strains				
	P. multocida subsp. *multocida* (n = 95)	*P. multocida* subsp. *septica* (n = 21)	*P. canis* (n = 28)	*P. stomatis* (n = 10)	*P. dagmatis* (n = 5)
Acid produced from:					
Maltose	0	0	0	0	5 (100)
Mannitol	95 (100)	21 (100)	0	0	0
Sorbitol	95 (100)	0	0	0	0
Trehalose	39 (41)	21 (100)	20 (71)	10 (100)	5 (100)
D-Xylose	45 (47)	21 (100)	28 (100)	0	0
Gas produced from glucose	0	0	0	0	3 (60)
Gelatin liquefaction	0	0	0	0	5 (100)
Ornithine decarboxylation	95 (100)	21 (100)	28 (100)	0	0
Tellurite tolerance	95 (100)	21 (100)	28 (100)	10 (100)	0
Urea hydrolysis	0	0	0	0	5 (100)

France). The bacteria were grown overnight on blood agar and suspended in saline to a density approximating a no. 5 McFarland turbidity standard. Two drops of the suspension was added to each cupule on the test strip. The tests were incubated at 37°C for 4 h, and then the reagents were added and the test results were recorded according to the manufacturer's instructions.

To test for extracellular lipase activity, the strains were grown overnight in 10 ml of fresh Mueller-Hinton broth (Difco) and then the bacteria were removed by sterile filtration. The presence of extracellular lipase activity in the cell-free supernatants was then analyzed. The lipase assay was modified from a method originally designed for the analysis of lipase activity involved in plasma lipoprotein metabolism. The assay substrate consisted of appropriate amounts of labeled and unlabeled trioleoylglycerol (Sigma Chemical Co., St. Louis, Mo.) emulsified by sonication, with 0.5% gum arabic as the emulsifier.

Gas chromatographic analysis of cellular fatty acids. Each strain was grown overnight in 10 ml of fresh Mueller-Hinton broth (Difco). The bacteria were washed twice in distilled water and freeze-dried. The pellets (5 to 15 mg) were heated in 1 ml of 2 M methanolic HCl at 90°C for 60 min for release of both ester- and amide-linked fatty acids. Hexane and distilled water (0.5 ml each) were added to each test tube, and the tubes were shaken to extract the fatty acid methyl esters. The organic phase was transferred to a new test tube and evaporated under a stream of dry nitrogen. Before gas chromatographic analysis, each sample was made up to approximately 30 μl.

The samples (1-μl aliquots) were analyzed with a Varian model 3500 gas chromatograph (Varian, Palo Alto, Calif.) equipped with a flame ionization detector by splitless injection onto a fused-silica capillary column (25 m by 0.2 mm) coated with SE-30. The temperatures of the injector and detector were 250 and 280°C, respectively, whereas the oven temperature was programmed to rise from 140 to 280°C at 6°C/min. The fatty acid methyl esters were identified by retention time comparisons and by gas chromatography-mass spectrometry.

Toxin production. A total of 56 strains, comprising 34 *P. multocida* subsp. *multocida*, 11 *P. multocida* subsp. *septica*, 7 *P. canis*, and 4 *P. stomatis* strains, were tested for toxin production at the National Veterinary Laboratory, Copenhagen, Denmark. The organisms were grown overnight on 5% calf blood agar, and then the bacteria were harvested in 2 ml of sterile water. The suspensions were left for extraction at 37°C for 18 h. Testing for *P. multocida* toxin production in the bacterial suspensions was performed by sandwich enzyme-linked immunosorbent assay (ELISA) by using monoclonal antibodies as previously described (2). The ELISA included binding *P. multocida* toxin from supernatant in microtiter plates coated with monoclonal antibody P3F51 and then washing with phosphate-buffered saline–Tween 20 (0.1%) and detection by a noncompetitive biotin-conjugated antibody, P3F37.

RESULTS

Biochemical differentiation and distribution of *Pasteurella* species and subspecies. Among the 159 *Pasteurella* strains studied, the biochemical tests distinguished the following species and subspecies: *P. multocida* subsp. *multocida* (95 strains), *P. multocida* subsp. *septica* (21 strains), *P. canis* (28 strains), *P. stomatis* (10 strains), and *P. dagmatis* (5 strains) (Table 1). All 159 strains produced indole and reduced nitrate, and none produced acid from L-arabinose or dulcitol. Three *P. dagmatis* strains produced gas in small amounts from glucose within 48 h, while all five such strains showed delayed gelatinase reactions within 3 weeks of incubation (Table 1).

P. multocida **subsp.** *multocida* was recovered from all 5 blood specimens, from cerebrospinal fluid from 1 of the 2 patients with meningitis, from peritoneal fluid from 1 patient with peritonitis, from 82 patients with wound infections or abscesses following cat or dog bites (63 and 19 cases, respectively), from 3 patients with wound infections who had only been licked by their dogs, and from cut wounds in 3 patients without evident connections with animals.

P. multocida subsp. *septica* was recovered from the cerebrospinal fluid of 1 meningitis patient and from 20 patients with 20 wound infections or abscesses, all associated with cat bites or scratches or with cats licking injured skin. *P. multocida* subsp. *multocida* was concomitantly recovered from three of the patients with wound infections.

All 28 strains belonging to *P. canis* were recovered from patients with wound infections associated with dog bites.

The 10 *P. stomatis* strains were recovered from eight patients with wound infections and two with abscesses following cat or dog bites. In all of the wound infections, either *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, or *P. canis* occurred concomitantly.

P. dagmatis was recovered in a severe cellulitis case, in a case of a groin abscess and a case of a throat abscess, and in two cases of wound infection following dog bites. In the cases of wound infection, *P. multocida* subsp. *multocida* and *P. canis* were also recovered.

Enzymatic activity. All 159 *Pasteurella* strains tested degraded 2-naphthyl phosphate, 2-naphthyl butyrate, 2-naphthyl caprylate, L-leucyl-2-naphthylamide, and naphthol-AS-BI-phosphate efficiently, thus indicating production of alkaline phosphatase, acid phosphatase, esterase, esterase lipase, leucine arylamidase, and naphthol-AS-BI-phosphohydrolase. All strains belonging to *P. multocida* subsp. *septica*, *P. stomatis*, and *P. dagmatis* degraded 2-naphthyl-α-D-glucopyranoside, which indicates production of α-glucosidase, and so did those strains of *P. multocida* subsp. *multocida* and *P. canis* which produced acid from trehalose. None of the strains studied produced extracellular lipase.

Toxin production. Of the 56 strains studied, 2 were found to produce toxin in the *P. multocida* toxin ELISA. The strains which belonged to *P. multocida* subsp. *multocida* and *P. canis* had been isolated in cases of wound infection following a cat bite and a dog bite, respectively.

Gas chromatographic analysis of cellular fatty acids. The resulting chromatograms of the different *Pasteurella* species were very similar. All strains contained major amounts of tetradecanoic ($C_{14:0}$), hexadecenoic ($C_{16:1}$), hexadecanoic ($C_{16:0}$), and 3-hydroxytetradecanoic (3-OH-$C_{14:0}$) acids and minor amounts of octadecadienoic ($C_{18:2}$), octadecenoic ($C_{18:1}$), and octadecanoic ($C_{18:0}$) acids.

DISCUSSION

Humans acquire *Pasteurella* infection primarily through animal contact (8, 19). Most infections are associated with animal bites or scratches, but infections with unknown sources of acquisition occur (9, 11, 16). Respiratory tract and intra-abdominal infections have been associated with possible inhalation of the organism (19). In the present study, *Pasteurella* strains were recovered in four cases of infection (three cut wounds and one groin abscess) in patients without evident connections with animals. In another five cases, *Pasteurella* strains occurred in cases of wound infection in patients who lacked a history of animal bites or scratches but who all reported being licked by their pet animals on injured skin. The five septicemia patients all kept domestic animals, either a cat or a dog or both; three of these patients reported being scratched or licked, but not bitten, by their animals.

The 159 strains characterized in this study represented all *Pasteurella* strains recovered in our laboratory during a 3-year period. On the basis of the biochemical tests, all strains could be assigned to one of the recently described taxa (13).

The enzymatic activities of the *Pasteurella* strains studied did not distinguish among strains belonging to different species. However, irrespective of identification of species, all strains acidifying trehalose degraded 2-naphthyl-α-D-glucopyranoside, indicating production of α-glucosidase. None of the *Pasteurella* strains produced extracellular lipases. Such enzymes, which may be used as virulence factors, are produced by many gram-positive and some gram-negative species.

It was not possible to distinguish among strains of the different *Pasteurella* species on the basis of fatty acid composition. The results of the gas chromatographic analyses of cellular fatty acids were in general agreement with those of a previous study including *P. multocida* strains, in which it was concluded that the fatty acid composition of *P. multocida* exhibited a pattern closely related to that of most *Haemophilus* species but could be distinguished by its higher concentration levels of the C_{18} fatty acids (10).

Only two *Pasteurella* strains, belonging to *P. multocida* subsp. *multocida* and *P. canis*, were found to produce toxin. Toxigenic strains of *P. multocida* subsp. *multocida* have previously been recovered from a number of animals, including dogs and cats (14). The *P. multocida* toxin is considered the central etiological factor in atrophic rhinitis in pigs (3). Toxigenic *P. multocida* subsp. *multocida* strains have been isolated from humans, both from sputum and from the respiratory tract and in cases of septicemia (15). However, the basis of the action of the toxin is not known and its role in the pathogenesis of human disease has not been elucidated. In the present study, none of the *Pasteurella* strains recovered in the more severe cases of infection produced toxin, while the two toxin-producing strains were recovered in cases of minor wound infection.

P. multocida strains are recovered from most mammals, including humans and birds (13, 18). *P. multocida* subsp. *multocida* is predominant in nearly all hosts. Mutters and coworkers (13) concluded that their DNA binding data would allow them to classify strains formerly classified as *P. multocida* as three distinct species but that such a distinction would be pointless from the clinical point of view. Therefore, they proposed the division of *P. multocida* into three subspecies, *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, and *P. multocida* subsp. *gallicida*; this division may be useful for epidemiological purposes. Strains belonging to *P. multocida* subsp. *gallicida* have been isolated mostly from avian sources (7, 13). Tests for acidification of dulcitol and sorbitol delineate the three subspecies (13). *P. multocida* can cause serious invasive infections; cases of meningitis have been reported (12, 19). Bacteremia, which frequently occurs in the setting of serious infections, has been reported to accompany meningitis in half of the cases (19). In the present study, *P. multocida* subsp. *multocida*, which accounted for 60% of the 159 *Pasteurella* isolates, was recovered in all septicemia cases, in one case each of meningitis and peritonitis, and in cases of wound infection or abscess following animal bites, scratches, or licking or without any known connections with animals. Most *P. multocida* subsp. *multocida* strains (70%) were recovered in cases of infection associated with cats.

A greater prevalence of *P. multocida* subsp. *septica* in cats than dogs has been reported (1). All our *P. multocida* subsp. *septica* strains were recovered in cases of infection associated with cats, including one meningitis case. Biberstein et al., who recovered *P. multocida* subsp. *septica* from three of four cats with infections of the central nervous system, suggested that this organism, compared with other *Pasteurella* species, may possess a preferential affinity for the central nervous system (1).

There are two biotypes of *P. canis*: biotype 1 is found in the oral cavities of dogs, while biotype 2 has been isolated from calves (13). The 28 isolates of *P. canis* presented here all belonged to biotype 1 and were without exception associated with wound infections following dog bites. From five of the cases, another *Pasteurella* species was recovered. *P. stomatis* occurs in the respiratory tracts of cats and dogs. Our 10 *P. stomatis* strains were isolated in cases of wound infection and abscess following cat or dog bites, but *P. stomatis* was the sole *Pasteurella* species recovered only from the abscesses. Thus, the organism may have played a secondary role in the wound infections, in which it occurred concomitantly with *P. multocida* and *P. canis*.

P. dagmatis occurs in both cats and dogs and has been isolated in cases of human local and systemic infection following animal bites (13). In the present study, *P. dagmatis* was, together with either *P. multocida* subsp. *multocida* or *P. canis*, recovered in two cases of wound infection resulting from dog bites. The cellulitis patient had a house cat but did not recall any history of bites, scratches, or licking, while the patient with a throat abscess following tonsillectomy reported being licked on her hands by her dog almost every day. The groin abscess, from which only *P. dagmatis* was recovered, occurred in a patient without evident connections with animals.

This study reveals something of the differential occurrence of *Pasteurella* species and subspecies in human clinical specimens submitted for microbiological diagnosis to our laboratory. *P. multocida* subsp. *multocida* was the predominant taxon throughout, with *P. canis* and *P. multocida* subsp. *septica* being the next most common. This distribution was similar for each year of the 3-year study period. More than one *Pasteurella* species was recovered in 13 cases of wound infection following cat or dog bites. In such cases, the roles of the different *Pasteurella* species may be difficult to establish. With the exception of infections involving *P. canis*, the majority of the *Pasteurella* infections were associated with cat bites or scratches. In only 3% of the 146 cases were *Pasteurella* species recovered from infected patients without evident connections with animals.

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